

terminal threonines or serines on proteins may be selectively oxidized to aldehydes.

Small linker molecules may also be used to functionalize proteins and polysaccharides with amino-oxy groups. See, for example, Vilaseca et al.,

- 5 "Protein conjugates of defined structure: synthesis and use of a new carrier molecule," *Bioconj. Chem.* 4:515 (1993); and Jones et al., "Synthesis of LJP 993, a multivalent conjugate of the N-terminal domain of b2GPI and suppression of an anti-b2GPI immune response," *Bioconj. Chem.* 12:1012 (2001).

- As is known to those of ordinary skill in the art, amino-oxy, aminooxy,  
10 aminoxy, and oxy-amine are all synonymous terms.

#### F. Indirect Conjugation

As stated above, the conjugation between the first moiety and the second moiety may proceed either indirectly or directly. In certain instances, the process of combining a protein and a polysaccharide may lead to undesirable side effects.

- 15 In some cases, direct coupling can place the protein and the polysaccharide in very close proximity to one another and encourage the formation of excessive crosslinks between the protein and the polysaccharide. Under the extreme of such conditions, the resultant material can become very thick (e.g., in a gelled state).

- 20 Over-crosslinking also can result in decreased immunogenicity of the protein and polysaccharide components. In addition, the crosslinking process can result in the introduction of foreign epitopes into the conjugate or can otherwise be detrimental to production of a useful vaccine. The introduction of excessive crosslinks exacerbates this problem.

- 25 Control of crosslinking between the protein and the polysaccharide can be controlled by the number of active groups on each, concentration, pH, buffer

composition, temperature, the use of spacers and/or charge, and other means well-known to those skilled in the art.

For example, a spacer may be provided between the protein and polysaccharide in order to control the degree of crosslinking. The spacer helps maintain physical separation between the protein and polysaccharide molecules, and it can be used to limit the number of crosslinks between the protein and polysaccharide. As an additional advantage, spacers also can be used to control the structure of the resultant conjugate. If a conjugate does not have the correct structure, problems can result that can adversely affect the immunogenicity of the conjugate material. The speed of coupling, either too fast or too slow, also can affect the overall yield, structure, and immunogenicity of the resulting conjugate product. Schneerson et al., *Journal of Experimental Medicine*, 152:361 (1980).

#### G. Vaccine Compositions

This invention further relates to vaccines and other immunological reagents that can be prepared from the conjugates produced by the method in accordance with the invention. For example, to produce a vaccine or other immunological reagent, the conjugates produced by the method according to the invention may be combined with a pharmaceutically acceptable medium or delivery vehicle by conventional techniques known to those skilled in the art. Such vaccines or immunological reagents will contain an effective therapeutic amount of the conjugate according to the invention, together with a suitable amount of vehicle so as to provide the form for proper administration to the patient. These vaccines may include alum or other adjuvants.

Exemplary pharmaceutically acceptable media or vehicles include, for example, sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. Saline is a preferred vehicle when the pharmaceutical

composition is administered intravenously. Aqueous dextrose and glycerol solutions can be employed as liquid vehicles, particularly for injectable solutions. Suitable pharmaceutical vehicles are well known in the art, such as those described in E. W. Martin, Remington's Pharmaceutical Sciences.

5           The vaccines that may be prepared in accordance with the invention include, but are not limited, to Diphtheria vaccine; Pertussis (subunit) vaccine; Tetanus vaccine; *H. influenzae* type b (polyribose phosphate); *S. pneumoniae*, all serotypes; *E. coli*, endotoxin or J5 antigen (LPS, Lipid A, and Gentabiose); *E. coli*, O polysaccharides (serotype specific); Klebsiella, polysaccharides (serotype  
10 specific); *S. aureus*, types 5 and 8 (serotype specific and common protective antigens); *S. epidermidis*, serotype polysaccharide I, II, and III (and common protective antigens); *N. meningitidis*, serotype specific or protein antigens; Polio vaccine; Mumps, measles, rubella vaccine; Respiratory syncytial virus; Rabies; Hepatitis A, B, C, and others; Human immunodeficiency virus I and II (GP120,  
15 GP41, GP160, p24, others); Herpes simplex types 1 and 2; CMV (cytomegalovirus); EBV (Epstein-Barr virus); Varicella/Zoster; Malaria; Tuberculosis; *Candida albicans*, other candida; *Pneumocystis carinii*; Mycoplasma; Influenzae viruses A and B; Adenovirus; Group A streptococcus, Group B streptococcus, serotypes, Ia, Ib, II, and III; *Pseudomonas aeruginosa*  
20 (serotype specific); Rhinovirus; Parainfluenzae (types 1, 2, and 3); Coronaviruses; Salmonella; Shigella; Rotavirus; Enteroviruses; *Chlamydia trachomatis* and *pneumoniae* (TWAR); and *Cryptococcus neoformans*.

          The invention also relates to the treatment of a patient by administering an immunostimulatory amount of the vaccine. The term "patient" refers to any  
25 subject for whom the treatment may be beneficial and includes mammals, especially humans, horses, cows, pigs, sheep, deer, dogs, and cats, as well as other animals, such as chickens. An "immunostimulatory amount" refers to that

amount of vaccine that is able to stimulate the immune response of the patient for prevention, amelioration, or treatment of diseases. The vaccines of the invention may be administered by any suitable route, but they preferably are administered by intravenous, intramuscular, intranasal, or subcutaneous injection. For  
5 example, carbohydrate-based vaccines can be used in cancer therapy.

In addition, the vaccines and immunological reagents according to the invention can be administered for any suitable purpose, such as for therapeutic, prophylactic, or diagnostic purposes.

The invention also relates to a method of preparing an immunotherapeutic  
10 agent against infections caused by bacteria, viruses, parasites, fungi, or chemicals by immunizing a patient with the vaccine described above so that the donor produces antibodies directed against the vaccine. Antibodies may be isolated or B cells may be obtained to later fuse with myeloma cells to make monoclonal antibodies. The making of monoclonal antibodies is generally known in the art  
15 (see Kohler et al., *Nature*, 256:495 (1975)). As used herein, "immunotherapeutic agent" refers to a composition of antibodies that are directed against specific immunogens for use in passive treatment of patients. A plasma donor is any subject that is injected with a vaccine for the production of antibodies against the immunogens contained in the vaccine.

20

### EXAMPLES

#### **Example 1: Preparation of an Amino-Oxy Functionalized Protein**

The following example illustrates the preparation of an amino-oxy functionalized protein that can be conjugated to a polysaccharide. Bovine serum albumin (BSA) was used as a model protein.

25 Bis(amino-oxy)tetraethylene glycol was linked to carboxyl groups on bovine serum albumin (BSA) with carbodiimide. Monomer BSA was prepared as described in (Lees et al., *Vaccine* 14:190, 1996). Bis(amino-oxy)tetraethylene

glycol (85 mg) (prepared by Solulink™, MW 361) was made up in 850 µl of 0.5 M HCl. 5 N NaOH was added to adjust to a pH ~4.5. 1 ml of BSA mono (42.2 mg/ml in saline) was added. The reaction was initiated by the addition of 25 µl of freshly prepared EDC (1-(3-dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride, 100 mg/ml in water). After approximately 3 hours, the solution was dialyzed overnight against saline at 4°C. The solution was then made up to 4 ml with saline and concentrated with an Amicon Ultra 4™ centrifugal device (30 kDa cutoff) to ~0.5 ml, and was further desalted on a 1x15 cm G-10 column (Pharmacia) equilibrated with saline. The void volume fraction was then concentrated to ~1 ml using the Amicon Ultra 4™ device. Using the BCA assay (Pierce Chemical Co), the protein concentration was estimated to be 34 mg/ml BSA. Trinitrobenzene sulfonic acid assay gave an intense red/orange, indicating the presence of amino-oxy group.

#### **Example 2: Preparation of an Amino-Oxy Derivatized Polysaccharide**

The following example illustrates the preparation of any amino-oxy functionalized polysaccharide that can be conjugated to a protein, peptide, or hapten.

Pn14 (10 ml at 5 mg/ml in water) was activated by the addition of 40 mg of CDAP (100 mg/ml stock in acetonitrile), followed by triethylamine to raise the pH to 9.4. After approximately 2.5 minutes, 4 ml of 0.5 M hexanediamine (pH 9.4) was added. The reaction was permitted to proceed for about 2 hours. Excess reagent was then removed by dialysis against saline to yield amino-Pn14.

Amino-Pn14 was then reacted with excess NHS bromoacetate at pH 8 and dialyzed against saline in the dark at 4°C. The bromoacetylated Pn14 was concentrated by pressure filtration and then dialyzed against water.

Amino-oxy cysteamine was prepared from bis amino-oxy cystamine by TCEP reduction followed by ion exchange on a Dowex 1X-8 column as follows:

Bis(amino-oxy)cystamine (obtained from Solulink) was made up in 50% NMP/water at 0.1 M. TCEP was made up in water at 0.5 M and 3x molar equivalents of 1 M sodium bicarbonate was added. A 1.5 molar excess of TCEP was combined with Bis(AO)cystamine, and adjusted to pH ~7 with sodium carbonate. After 10 minutes, the mixture was diluted 5-fold into 10 mM bistris at pH 5. The reaction mixture was applied to a 1x3 cm Dowex 1-x8 column that had been washed with 1 M NaCl and equilibrated with 10 mM bistris, pH 5. The reduced amino-oxy cysteamine is found in the flow through of the column.

Amino-oxy cysteamine was added to the bromoacetylated Pn14 and reacted at pH 8 in the dark. The reaction mixture was then concentrated, diafiltered, and then dialyzed against water.

Pn14 concentration was determined to be 9.1 mg/ml by the resorcinol/sulfuric acid method. Using the TNBS assay and amino-oxy acetate as the standard, the amino-oxy concentration was estimated at 0.74 mM, resulting in about 8 amino-oxy groups per 100 kDa of polysaccharide.

### **Example 3: Preparation of a BSA-Dextran Conjugate**

The following example illustrates the preparation of a conjugate vaccine using an amino-oxy functionalized protein and an oxidized polysaccharide. Specifically, the amino-oxy functionalized BSA prepared in Example 2 was linked to oxidized dextran.

Dextran was oxidized using sodium periodate as follows: A 10 mg/ml solution of T2000 dextran (Pharmacia) was made to 10 mM in sodium acetate, pH 5 and then 10 mM sodium periodate (from a 0.5 M stock in water), and incubated at room temperature in the dark. At 1, 5, 10 and 15 min, an aliquot was removed, quenched by the addition of glycerol, and dialyzed against water in the dark. The final concentration of dextran was determined to be about 4.5 mg/ml.

The protein was conjugated to the polysaccharide as follows: 110  $\mu$ l of each oxidized dextran preparation (1-15 min oxidation) was combined with 15  $\mu$ l BSA- amino-oxy (0.5 mg each). After an overnight reaction in the dark at room temperature, the samples were analyzed by SDS PAGE (4-12% gradient gel, NuPAGE, Invitrogen). With reference to Figure 1, lanes are conjugates prepared with (A) dex ox 1 min; (B) dex ox 5 min; (C) dex ox 10 min; (D) dex ox 15 min; BSA- amino-oxy only. It is evident that each of the conjugation reactions resulted in high molecular weight material that did not enter the gel. Essentially no unconjugated protein is evident, indicating a high degree of conjugation.

10 The four conjugates were pooled & fractionated on a S-400HR™ gel filtration column (1x60 cm), equilibrated with saline. The void volume fractions were pooled and assayed for protein and polysaccharide. It was determined that the pool contained 0.21 mg/ml BSA and 0.27 mg/ml dextran. At least 50% of the initial protein and polysaccharide were recovered. Thus, the amino-oxy-protein with oxidized polysaccharide yielded soluble conjugate in excellent yield.

#### **Example 4: Preparation of AO-Functionalized TT.**

The following hypothetical example illustrates the preparation of Tetanus toxoid derivatized with amino-oxy groups using a two-step method.

1 ml tetanus toxoid (10 mg/ml) in 2 M NaCl is made to pH 8 by the addition of 50  $\mu$ l 1 M HEPES, pH 8. The protein is bromoacetylated by the addition of 7  $\mu$ l of 0.1 M NHS bromoacetate. After a 1 hour incubation, 2  $\mu$ moles of aminocysteamine is added. After an overnight reaction, excess reagent is removed by dialysis against 2 M NaCl.

The protein concentration is determined using the BCA assay (Pierce Chemical) and the presence of the amino-oxy group confirmed using TNBS.

**Example 5: Preparation of amino-oxy-derivatized BSA using a two-step method**

**Bromoacetylation of BSA:**

5           4.1 ml of monomeric BSA (48.5 mg/ml) was made to pH 8 by the addition of 400  $\mu$ l 1 M HEPES, pH 8 and 5.5 ml water. 1 ml of 0.2 M NHS bromoacetate (ProChem) in NMP was slowly added while vortexing. After an overnight reaction at room temperature in the dark, the solution was dialyzed against saline for 2 days, centrifuged and filtered. 10.6 ml of BSA at 15.3 mg/ml was obtained.

10           **Preparation of Amino-oxy cysteamine:**

          51.5 mg of Bisaminocysteamine was added to a solution of 56 mg TCEP made up in 1.1 ml 1 M sodium carbonate, 586  $\mu$ l DMSO, and 586  $\mu$ l water. After 15 minutes, the TCEP was removed on a 1x5 cm Dowex 1x-8 column, equilibrated with 10 mM Bistris, pH 6. The DTNB positive flow thru was pooled  
15   and found to be 22.6 mM thiol.

          6 ml was added to the bromoacetylated BSA and the pH adjusted to 8. The reaction was allowed to proceed overnight in the dark, and was then dialyzed for 2 days at 4° C against multiple changes of saline. The amino-oxy BSA was determined to be about 8.6 mg/ml. Reaction of an aliquot with TNBS at pH 8 gave  
20   an orangish color, indicating the presence of the amino-oxy group.

**Example 6: Use of CDAP to Prepare Amino-Oxy Derivatized Polysaccharide and Amino-oxy Conjugates**

          This experiment illustrates the use of CDAP to prepare amino-oxy derivatized polysaccharide and amino-oxy conjugates. It illustrates how chemistry  
25   other than oxidation can be used to functionalize a polysaccharide with amino-oxy groups.



### I. Preparation of an amino-oxy derivatized polysaccharide using CDAP

#### chemistry

A solution of bifunctional amino-oxy reagent was prepared by solubilizing 29 mg of bis-amino-oxy acetate (ethylene diamine) (prepared by Solulink™) in 5 200 µl 1 M NaAc, pH 5. Dextran was activated using CDAP chemistry as follows. To a solution of 0.5 ml T2000 dextran at 10 mg/ml in water, 25 µl of CDAP (100mg/ml acetonitrile) was added and 30 seconds later the pH was raised by the addition of 25 µl 0.2 M triethylamine (TEA) and three 5 µl of TEA neat.

At 2.5 minutes, the pH was reduced by the addition of 100 µl 1 M NaAc, pH 10 5. 200 µl of the BisAO solution was then added. After ~30 minutes reaction, the solution was desalted on a 1 x 15 cm P6DG column (BioRad) equilibrated with NaAc buffer (10 mM NaAc, 150 mM NaCl, 5 mM EDTA, pH 5). The desalted polysaccharide was estimated at 1.7 mg/ml dextran, using the resorcinol assay, and about 11 amino-oxy groups/100 kDa dex using a TNBS assay.

### II. Preparation of oxidized ovalbumin

To a 0.4 ml solution of ovalbumin (14.4 mg) (OVA), 10 µl of 1 M sodium acetate, pH 5 was added, followed by the addition of 10 µl 0.5 M sodium periodate (in water). After a 15 minutes incubation at room temperature in the dark, the reaction was quenched with the addition of a few drops of 50% glycerol. The 20 reaction mixture was then dialyzed in the dark against NaAc buffer. By adsorption at 280 nm, the concentration of oxidized ovalbumin ("OVA(ox)") was 6.6 mg/ml.

### III. Preparation of conjugates and controls

The following solutions were prepared and each was incubated overnight at room temperature in the dark:

- 25 A. 500 µl Dex AO (0.85 mg) + 75 µl OVA(ox) + 100 µl 1 M NaAc pH 5.  
B. 250 µl Dex AO (0.043 mg) + 37.5 µl NaAc buffer + 50 µl 1 M NaAc  
C. 250 µl NaAc buffer + 37.5 µl OVA(ox) + 50 µl 1 M NaAc.

Each was then assayed by SDS PAGE and SEC HPLC. Only sample A contained high molecular weight (HMW) material, with ~ 20% of protein conjugated, as estimated by SEC HPLC. Neither B nor C indicated any HMW material by SEC HPLC or SDS PAGE.

**5 Example 7: Use of Cyanogen Bromide to Label Polysaccharide with a Bis-Amino-Oxy Reagent.**

This prophetic example demonstrates the derivatization of a polysaccharide with an amino-oxy reagent using cyanogen bromide (CNBr).

Polysaccharide (e.g., Pn-14) is made up at 10 mg/ml in water, and is  
10 treated with CNBr at 1 mg per mg of polysaccharide at pH 10.5 for 6 minutes in a pH-stat. The reaction mixture is then reduced to ~pH 7 by the addition of 0.5 M bis-amino-oxy reagent (e.g., bis-AO(EDA). After an overnight reaction, the solution is dialyzed into water and assayed for amino-oxy groups with TNBS, and for carbohydrates with the resorcinol assay. This amino-oxy derivatized  
15 polysaccharide is used for conjugation with a carbonyl-containing protein.

According to another embodiment, the CNBr-activated polysaccharide can be reacted with amino-oxy acetate. This will result in a polysaccharide functionalized with carboxyl groups. The carboxyl groups can then be further functionalized and indirectly or directly linked to proteins (with, for example,  
20 carbodiimide).

**Example 8: Conjugation of Amino-Oxy Derivatized Protein with Oxidized Polysaccharide**

This example illustrates the preparation of amino-oxy derivatized protein with the functionalization occurring on the amines. This amino-oxy derivatized  
25 protein is then covalently linked to the clinically relevant polysaccharides *Neisseria meningitidis* A and C.

I. Functionalization of protein with amino-oxy groups of a protein on its amines (protein with pendent amino-oxy groups on amines)

Amines on the protein are bromoacetylated and then reacted with a thiol-amino-oxy reagent to produce a protein with pendent amino-oxy groups.

- 5 Bis(amino-oxy acetate)cystamine 2HCl was prepared by Solulink.<sup>TM</sup> Monomeric BSA was at 42.2 mg/ml. NHS bromoacetate was obtained from Prochem and made up at 0.1 M in NMP (N-methyl-2-pyrrolidone). The amino-oxy protein was prepared as follows. In each of 2 tubes, a solution of 0.5 ml of BSA (21.1 mg) and 250  $\mu$ l H<sub>2</sub>O + 100  $\mu$ l 1 M HEPES, pH 8 was prepared. One tube was reacted with  
10 a 30 fold molar excess of NHS bromoacetate (93  $\mu$ l) and the other at a 10 fold molar excess (31  $\mu$ l).

After about 1 hr, each was made up to 15 ml with sodium acetate buffer (10 mM NaAc, 0.15 M NaCl, 5 mM EDTA, pH 5) and concentrated to about 200  $\mu$ l using an Amicon Ultra 15<sup>TM</sup> device (30 kDa cutoff).

- 15 Amino-oxy acetate cysteamine was prepared as follows:

To a solution of 9.8 mg of Bis(AOAc)cystamine (prepared by Solulink<sup>TM</sup>) in 114  $\mu$ l 1 M sodium acetate + 114  $\mu$ l NMP, 22.8  $\mu$ l of 0.25 M TCEP in 1 M HEPES, pH 8 was added as a reducing agent. After 1 hour, the partially reduced amino-oxy thiol reagent was added to each of the bromoacetylated BSA preparations,  
20 the pH was adjusted to about pH 8 and the reaction allowed to proceed overnight in the dark at 4°C.

Each was desalted using the Amicon Ultra 15<sup>TM</sup> device by making volume up to 15 ml with NaAc buffer and centrifuging. The desalting process was repeated four times. The final volume was about 200  $\mu$ l and was then made up to  
25 about 1 ml with NaAc buffer. This product was termed BSA-S-AO. By adsorbance at 280 nm, the 30x prep was determined to be 29.8 mg/ml and the 10x prep 24.8 mg/ml.

## II. Preparation of oxidized *Neisseria meningitidis* polysaccharide A and C (Neiss PsA and Neiss PsC)

Neiss PsA and PsC were solubilized overnight at room temperature at 10 mg/ml in water and then stored at 4°C. 50 µl of 1 M sodium acetate, pH 5, was added to 1 ml of each polysaccharide solution, followed by the addition of 25 µl 0.5 M sodium periodate (0.5 M in water). After 10 minutes in the dark at room temperature, each was dialyzed 4 hours against 4 l water. Each was then made up to 4 ml with water and further desalted using an Amicon Ultra 4™ device (30 kDa cutoff). Using the resorcinol assay, the oxidized Neiss PsA was determined to be 12.1 mg/ml and the oxidized Neiss PsC was 17.8 mg/ml.

## III. Conjugation of BSA-S-AO with oxidized Neiss PsA and PsC

The following mixtures of BSA-S-AO and oxidized PsA and PsC were prepared.

Conjugate	µl Ps	µl BSA	1 M NaAc pH 5
BSA10x-PsA	175 µl (2.1 mg)	134 µl (4 mg)	25 µl
BSA30x-PsA	175 µl (2.1 mg)	161 µl (4 mg)	25 µl
BSA10x-PsC	175 µl (3.1 mg)	134 µl (4 mg)	25 µl
BSA30x-PsC	175 µl (3.1 mg)	161 µl (4 mg)	25 µl

After an overnight reaction at room temperature in the dark, conjugates were assayed by SDS PAGE using a Phast gel (8-25%)(Pharmacia) under reducing conditions. With reference to Figure 2, from left to right the lanes are BSA30x-PsA, BSA30x-PsC, BSA30x, BSA10x -PsA, BSA10x-PsC, BSA10x. It is seen that there is a significant amount of high molecular weight materials that did not enter the gel, indicating that conjugation of the protein to the polysaccharide occurred.

The PsA conjugates were pooled and fractionated by gel filtration on a S-400HR column (1x60 cm, Pharmacia), equilibrated with saline. Similarly, the PsC conjugates were pooled and fractionated. Approximately 1 ml fractions were collected and assayed for protein (by absorbance) and for carbohydrate using the resorcinol assay. The results are provided in Figure 3.

For the PsC conjugate, tubes 18-22 were pooled and for the PsA conjugate, tubes 19-23 were pooled and examined by SDS PAGE using reducing conditions.

With reference to Figure 4, the BSA-Neiss PsC conjugate is on the left and the PsA conjugate is next to it. On the right is the molecular weight standard. A small amount of free BSA is observed in each, indicating incomplete separation of the conjugated and free protein. Each contains a significant amount of conjugated high molecular weight material that did enter the gel.

#### **Example 9: Preparation of (BSA-Levulate)-Amino-Oxy-Pn14 Conjugate**

This example illustrates the reaction of an amino-oxy group with a ketone and shows that this can be used for the formation of conjugates and, more specifically, the preparation of (BSA-Levulate)-Amino-oxy Pn14.

NHS Levulate was obtained from Solulink and made up by solubilizing 5.1 mg in 100  $\mu$ l NMP. This was slowly added to a vortexed solution of 200  $\mu$ l BSA at 48.5 mg/ml, 200  $\mu$ l water, and 100  $\mu$ l 1 M HEPES, pH 8. After an overnight reaction, the mixture was diafiltered using an Amicon Ultra 15 device, (30 kDa cutoff). The final volume was 0.5 ml. This product is BSA-LEV

100  $\mu$ l of BSA-LEV was combined with 300  $\mu$ l of amino-oxy Pn14 (4.5 mg/ml Pn14) and incubated for several days in the dark. The conjugate and the individual components were assayed by SEC HPLC using a Superose 6 column (Pharmacia). The conjugate was then fractionated on an S400HR column. Protein was assayed using the Bradford dye method, and polysaccharide with the

resorcinol method. The high molecular weight fraction was found to contain 0.6 mg BSA/mg Pn14.

**Example 10: Preparation of Amino-oxy-BSA – Neisseria PsC Conjugate**

Neiss PsC was oxidized to create terminal aldehyde as generally described in Jennings & Lugowski *J. Imm.* 127:1011 (1981). SEC HPLC indicated the molecular weight of the PsC was significantly reduced.

After overnight conjugation of PsC and BSA-AO, analysis was conducted via SEC HPLC Superose 6 0.5 ml/min. The conjugate was fractionated on a 1x60cm S200HR column, equilibrated 10 mM sodium acetate, 150 mM NaCl, 2 mM EDTA, pH 5. It was determined by both SEC analysis and gel filtration that most of BSA was conjugated. The high molecular weight peak was analyzed for protein and carbohydrate and determined to contain 0.2 mg BSA/mg PsC.

**Example 11: Preparation of amino-oxy-BSA – Neisseria PsA conjugate**

This example illustrates the preparation of a Neisseria PsA-BSA conjugate by way of functionalizing the protein with an amino-oxy group.

Neiss PsA was terminally reduced to an alditol with NaBH<sub>4</sub> and then oxidized to create terminal aldehyde as generally described in Jennings & Lugowski *J. Imm.* 127:1011 (1981).

Neisseria PsA was solubilized in water at 20 mg/ml for 15 min. To 1 ml of the solubilized polysaccharide, 10 mg of sodium borohydride was added. The pH was maintained to about 8-9. After 1 hour, 100 µl of 1 M NaAc was added, and the pH was adjusted to 5. The reduced PsA was desalted on a 1x15 cm G10 column, equilibrated with saline, and the void volume fraction concentrated with an Amicon Ultra 4 (10 kDa cutoff device) to about 1 ml. 20 mg of solid sodium periodate was added, along with 100 µl 1 M sodium acetate at pH 5. After a 15 minute oxidation in the dark at room temperature, the reaction was quenched by the addition of a drop of glycerol and then desalted on 1x15cm G10 column

equilibrated with 10 mM NaAC, 150 mM NaCl and 2 mM EDTA, pH 5 (acetate buffer). The void volume was pooled and found to be positive in the BCA assay, indicating the presence of reducing sugar. The material was diafiltered and concentrated with an Ultra 4 device into acetate buffer.

- 5           Both the amino-oxy BSA and the PsA(red/ox) were examined by SEC HPLC. The molecular weight of the PsA was markedly reduced by the reduction/oxidation process.

### Conjugation

- In the conjugation step, 150  $\mu$ l amino-oxy-BSA at 6 mg/ml was combined  
10   with 50  $\mu$ l PsA (red/ox) and 25  $\mu$ l 1 M NaAc at pH 5.

After an overnight incubation in the dark at 4° C, the conjugate was analyzed by SEC HPLC (Superose6, saline, 0.5 ml/min). It was seen that the PsA contributed very little absorbance and the AO-BSA increased in molecular weight on conjugation.

- 15           The conjugate was fractionated on a 1x60cm S200HR gel filtration column and the high molecular weight fraction assayed for protein and PsA and was found to contain 0.4 mg BSA/mg PsA.

- Conclusion: The reduction/oxidation method works well to create aldehydes that can be linked to amino-oxy-protein. PsA was probably hydrolyzed  
20   during the NaBH<sub>4</sub> step, which is at elevated pH.

### Example 12: Preparation of PRP(ox)-BSA-AO Conjugate

#### 1. Oxidation of PRP Hib

- 22.7 mg PRP Hib was made up at 10 mg/ml in water, and combined with 100  $\mu$ l 1 M NaAc and 46  $\mu$ l 0.5 M sodium periodate. The reaction proceeded in  
25   the dark and on ice for 15 minutes, and was then quenched with 50% glycerol. The reaction mixture was diafiltered into water with an Amicon Ultra 4 (10 kDa cutoff) device, 4 x 4ml, final volume was approximately 1 ml. A resorcinol assay

was conducted at 10 mg/ml. The sample was positive in the BCA assay, indicating the presence of aldehyde.

## 2. Conjugation amino-oxy BSA

AO-S-BSA was provided at 15 mg/ml. 667  $\mu$ l BSA-S-AO 10 mg was  
5 combined with 100  $\mu$ l 1 M sodium acetate, at pH 5, and approximately 1 ml PRP(ox), and the reaction was permitted to proceed overnight in the dark. It was then quenched by the addition of 50  $\mu$ l 0.25 M amino-oxy acetate.

## 3. Assay by SEC Superose6 prep grade HR10/30, equilibrated PBS 0.5 ml/min, OD 220.

10 Here, 0.5 ml conjugate was fractionated on 1x60 cm S200HR, and equilibrated PBS. All fractions eluted before BSA, indicating higher MW.

Fraction	BSA/mg Hib PRP (mg)
9	0.7
10	0.6
11	0.4
12	0.5

## **Example 13: Preparation of BSA-Pn14 Conjugate via Oxidation of Glycidic Acid**

15 This example illustrates a protocol whereby glycidic acid was added to amines on BSA using carbodiimide. The glycidic acid on the protein was then oxidized and reacted with amino-oxy-Pn14.

### I. BSA-Glycidic acid

Monomeric BSA and glycidic acid (obtained from Fluka Chemical) were  
20 combined to a final concentration in water of 12.5 mg/ml and 28 mg/ml, respectively. The pH was adjusted to about 5 and 220  $\mu$ l of 100 mg/ml EDC in water was added. The pH is kept at about 5 for approximately 1.5 hours, and the



reaction was quenched by the addition of .025 ml 1 M sodium acetate at pH 5.

The reaction mixture is then dialyzed against saline at 4°C overnight.

## II. Oxidation of BSA-glycidic acid

100 µl of 1 M sodium acetate at pH 5 was added to 1 ml of BSA-glycidic  
5 acid (7.8 mg/ml), followed by 25 µl of 0.5 M sodium periodate in water. After 10  
minutes in the dark, glycerol was added to quench the reaction and excess  
reagent removed using an Amicon Ultra centrifugal device with a 30 kDa cutoff.  
The final volume was about 400 µl.

100 µl of the oxidized BSA-glycidic acid was combined with 250 µl of  
10 amino-oxy Pn14 at 9.3 mg/ml along with 50 µl 1 M sodium acetate at pH 5. An  
aliquot was evaluated by SEC HPLC (Superose 6 0.5 ml/min, PBS). After an  
overnight reaction, another aliquot was assayed in the same way. It was seen  
that a significant portion of the absorbance eluted at the void volume (~15  
minutes), indicating that the protein was linked to the high molecular weight Pn14.

15 Following gel filtration on an S400HR column (Pharmacia), the high  
molecular weight fraction was determined to contain 0.3 mg BSA/mg Pn14. This  
ratio is similar to that determined from the percentage of conjugated high  
molecular weight protein in the above chromatogram.

Thus, the method of linking glycidic acid to protein using carbodiimide  
20 provides a way to create aldehydes on proteins that can be subsequently linked to  
amino-oxy groups.

### **Example 14: Use of Amino-Oxy Chemistry to link BSA to Dextran**

This example illustrates the coupling of an oligosaccharide via its reducing  
end to amino-oxy derivatized protein.

25 T40 dextran was made up at 100 mg/ml in water. The number of reducing  
ends was estimated using the BCA assay with glucose as the standard. It was

found that there were 3.5 mM reducing ends/100 mg/ml T40 dextran, so the average molecular weight was taken to be approximately 28,000 kDa

5 mg amino-oxy BSA containing ~8 amino-oxy/BSA was combined with 2 ratios of T40 dextran at pH 5.

5 (A) 830  $\mu$ l BSA-AO 15.3 mg/ml was combined with 620  $\mu$ l T40 dextran at 100 mg/ml and 100  $\mu$ l 1 M NaAc at pH 5.

(B) 830  $\mu$ l BSA-AO 15.3 mg/ml was combined with 3.1 ml T40 dextran at 100 mg/ml and 500  $\mu$ l 1 M NaAc at pH 5.

Solutions were reacted at room temperature in the dark for 1 week, and  
10 then assayed by SEC HPLC.

. Both conjugates eluted much earlier than BSA-AO, indicating that their molecular weight has increased. Conjugates were then fractionated by anion ion exchange (IEX). Consistent with the SEC profile, the higher the molecular weight, the lower ionic strength the conjugate eluted. IEX elution fractions were analyzed  
15 for the ratio of carbohydrate to protein and plotted on both a weight and mole ratio (using 28 kDa MW for the T40 dextran)

The peak fraction from each IEX elution was analyzed by SEC HPLC (Superose 6 1 ml/min). The absence of monomeric BSA and the increasing MW for high vs. low ratio T40dex/BSA conjugates. SDS PAGE confirmed the high  
20 molecular weight nature of the conjugate IEX eluants.

#### **Example 15: Use of Amino-Oxy Chemistry to Link Oligosaccharide and Protein**

This example demonstrates the use of amino-oxy chemistry to link an oligosaccharide indirectly via its reducing end to a protein. A general description  
25 of the protocol is as follows. The reducing end of T40 dextran (~40 kDa MW) was reacted with the amino-oxy group of amino-oxy acetate to create a dextran with a single carboxyl group on one end. This carboxy group was then converted to an

amine by reaction with ethylenediamine and carbodiimide. The amine-tipped dextran was then thiolated and reacted with maleimide-derivatized BSA, to create a conjugate consisting of a protein with "threads" of carbohydrate extending from it.

5        I. Addition of amino-oxy acetate to the reducing end of dextran

850 mg of T40 dextran (Pharmacia) was solubilized in 850  $\mu$ l of water overnight at room temperature.

235 mg of amino-oxy acetate was solubilized in a mixture of 850  $\mu$ l DMSO and 500  $\mu$ l 1 M sodium acetate, pH 5 and combined with the T40 dextran solution.

10       An additional 500  $\mu$ l of DMSO was added to make the solution approximately 50% DMSO. After an incubation at about 68°C for about 6 hours, the solution was extensively dialyzed against water. The product was dextran containing a single carboxyl group on its reducing end.

1.8 g of ethylenediamine 2HCl was added to the solution (approximately 22 ml) and the pH adjusted to approximately 5 with 1 N NaOH. 220 mg of EDC (1-(3-dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride) was added and the pH maintained at about 5 for 3 hours. The reaction was then quenched by the addition of 1 M sodium acetate, pH 5, dialyzed against saline, and concentrated using an Amicon Ultra 15™ (10 kDa cutoff). It was then further dialyzed against  
15       saline and then against water.  
20

The product was assayed for amines using TNBS and for carbohydrate using the resorcinol assay. It was determined that there were approximately 0.45 amines per 40,000 kDa of dextran. This product was dextran containing a single amine group on its reducing end and was termed NH<sub>2</sub>-AOAc-T40 dextran. Using  
25       the resorcinol assay, the solution was determined to have a concentration of about 119 mg/ml dextran. T40 dextran consists of a distribution of molecular

weights, which makes it difficult to determine the actual degree of substitution of the reducing ends of the polymers.

## II. Thiolated dextran and maleimide- BSA

Maleimide-derivatized BSA was prepared as follows: GMBS (40  $\mu$ l of a 0.1 M stock in NMP) was added to a solution of 200  $\mu$ l of monomeric BSA (42.2 mg/ml), 50  $\mu$ l 0.75 M HEPES, 5 mM EDTA at pH 7.3, and 100  $\mu$ l water. After a 2 hour reaction, the pH was reduced by the addition of 100  $\mu$ l 1 M sodium acetate, at pH 5. The solution was desalted using an Amicon Ultra 4™ (30 kDa cutoff) ultrafiltration device and 10 mM NaAc, 0.15 M NaCl, 5 mM EDTA, pH 5.

The NH<sub>2</sub>-AOAc-T40 dextran was thiolated using SPDP as follows: 0.5 ml of the NH<sub>2</sub>-AOAc-T40 dextran was combined with 100  $\mu$ l of 1 M HEPES, pH 8 and 100  $\mu$ l of 0.1 M SPDP were added. After approximately 2 hours, 50  $\mu$ l of 0.1 M EDTA pH 5 was added, followed by 100  $\mu$ l of 1 M sodium acetate, pH 5 and 50  $\mu$ l of 0.5 M dithiothreitol in water. After a 1 hour incubation, the solution was dialyzed into sodium acetate buffer overnight at 4°C.

The thiol tipped T40 dextran and the maleimide derivatized BSA were combined (a small aliquot of the BSA-maleimide was saved for analysis). After an overnight reaction, one half the mixture (about 1 ml) was fractionated by gel filtration using a 1x60 cm S-400HR column, equilibrated with saline. For comparison, a mixture of 100  $\mu$ l BSA monomer (42.2 mg/ml), 300  $\mu$ l T40 dextran AOAc, and 0.5 ml saline was similarly fractionated on the same gel filtration column. Fractions (about 1 ml) were analyzed for protein by absorbance at 280 nm and for dextran using the resorcinol assay.

With reference to Figures 5A-D, it is evident that the protein and dextran are eluting earlier from the column when in the T40 dextran AOAc-thiol-maleimide BSA conjugate than when the components are mixed. This indicates that a

conjugate of higher molecular weight has been formed. Furthermore, the ratio of dextran to protein increased.

The column fractions were further analyzed by SDS PAGE, with the results provided in Figure 6. From left to right, MW marker, conjugate fractions 18,  
5 20,22,24,26, mixture fractions # 24,26,28, 30, unfractionated conjugate, starting BSA-maleimide.

It is evident that the unfractionated conjugate contains only a small proportion of free protein, indicating that the conjugate was formed in high yield. No high molecular weight protein is evident in the mixture fractions. Only  
10 conjugate and essentially no free protein is evident in the conjugate fractions. This confirms that a conjugate formed in high yield.

**Example 16: Preparation of BSA-Pn14 Conjugates via Glycidic Acid and Amino-Oxy Derivatized Pn-14.**

The following example is illustrative of the preparation of a conjugate using  
15 an aldehyde-substituted protein.

I. In situ synthesis of NHS ester of glycidic acid using TSTU and addition to BSA

In this step, 7.9 mg of glycidic acid hemi-calcium salt monohydrate (MW 143) was solubilized in 110  $\mu$ l NMP. This was combined with 200  $\mu$ l of 0.5 M  
20 TSTU (Novachem) in NMP, and 100  $\mu$ l of triethylamine, and was added to 1 ml of 24 mg/ml BSA. The pH was adjusted to pH 8. After approximately 2 hours, the mixture was dialyzed on 2 x 1 liter saline. The number of free amines on BSA was determined using TNBS. For the control, the number was 33.2  $\text{NH}_2$ /BSA. For glycidic acid/TSTU/BSA, the number was 25  $\text{NH}_2$ /BSA. These results lead to  
25 the conclusion that BSA was labeled with about 8 glycidic acid units /BSA

## II. Oxidation of Functionalized BSA

A 5 mg aliquot was made up with 25 mM NaAc at pH 5, and 25 mM sodium periodate. The reaction was allowed to proceed in the dark at room temperature for 15 minutes, after which a drop of glycerol was added to quench the reaction.

- 5 The mixture was fractionated S200HR, pool main peak & concentrate.

## III. Preparation of BSA(ox)-AO-Pn14 Conjugate

In this step, 444 µl of amino-oxy functionalized Pn14 was combined with 0.4 ml BSA(ox) made up at 10.1 mg/ml, and 100 µl 1 M NaAc at pH 5, and reacted overnight at room temperature. The Fractionate by gel filtration S400HR  
10 1x60 cm saline + 0.02% azide SEC HPLC indicated that oxidation of the Glycidic acid/TSTU/BSA caused polymerization of the BSA.

Also observed was the progressive increase in the high molecular weight peak, indicating that conjugation was increasing with time. The AO-Pn14 alone had minimal absorbance.

## **15 Example 17: Preparation of Mercaptoglycerol-Bromoacetate BSA**

This example illustrates a process for preparing a BSA(mercaptoglycerol(ox))-AO-dextran conjugate.

### Preparation of bromoacetylated BSA

500µl monomeric BSA (48mg/ml) was combined with 500µl 1 M HEPES, at  
20 pH 8, and 25 µl 0.1 M NHS bromoacetate in NMP. For the control, 250 µl BSA was combined with 250 µl HEPES and 12 µl NMP

After approximately 1 hour, each was desalted into saline using an Amicon Ultra 4 (30 kDa cutoff) device. The final volume was 450 µl BSA-bromoAc, and 300µl BSA control

25 Next, 50 mM mercaptoethanol and 50 mM mercaptoglycerol were prepared in water.

Preparation E: 225  $\mu$ l BSA-BromoAc was combined with 100  $\mu$ l 1 M HEPES at pH 8 and 50  $\mu$ l of 50 mM mercaptoglycerol.

Preparation F: The BSA control was combined with 100  $\mu$ l 1 M HEPES to pH 8 and 50  $\mu$ l 50 mM mercaptoglycerol

5 Preparation G: 225  $\mu$ l BSA- BromoAc was combined with 100  $\mu$ l 1 M HEPES at pH 8, and 50  $\mu$ l 50 mM mercaptoethanol.

After 30 minutes, each was desalted with Amicon Ultra using NaAc buffer (10 mM NaAcetate, 150 mM NaCl, 5 mM EDTA, pH 5). Final volume was 0.5 ml

Each was then made up in 10 mM sodium periodate from a freshly  
10 prepared 0.5 M stock and incubated for 10 minutes at 4°C in the dark, and then quenched by the addition of glycerol and desalted using the Amicon Ultra device and washed into NaAc buffer. By OD 280, each was determined to be about 20 mg/ml BSA.

Preparation E should contain BSA-aldehyde, Preparation F was not labeled  
15 with the bromoacetate, and so it could not react with the mercaptoglycerol. Thus, it should not contain aldehydes. Preparation G would have pendent mercaptoethanol, which does not oxidize, so it should not contain aldehydes.

315  $\mu$ l of Amino-oxy dextran, at 15.9 mg/ml, was combined with 250  $\mu$ l of each BSA preparation, and incubated overnight at room temperature in the dark.

20 Each was then fractionated by gel filtration on a S400HR 1x60cm equilibrated with saline. The high molecular weight fraction was analyzed for protein and dextran.

The results indicated that only BSA containing the oxidized mercaptoglycerol formed a conjugate, and this was confirmed by the  
25 protein/dextran ratio of the high molecular weight fraction.

E BSA-mercaptoglycerol(ox) + AO-dex	0.97 mg BSA/mg dex.
F BSA control (ox) + AO-dex	0.1 mg BSA/mg dex.

G BSA-mercaptoethanol(ox) + AO-dex      0.1 mg BSA/mg dex.

#### Example 18: Linking of a Protein to a Polysaccharide via Oxime Formation

The following example illustrates the linking of a protein via its N-terminal group to a polysaccharide via oxime formation.

5      N-terminal threonine of lysostaphin was oxidized and derivatized with a bis-amino-oxy reagent. Oxidation of the protein was performed as generally described in Gaertner & Offord, "Site-specific attachment of functionalized poly(ethylene glycol) to the amino terminus of proteins," *Bioconjugate Chem.* 7:38 (1996). Lysostaphin a 27 kDa protein was produced in lactococcus.

#### 10      Trial No. 1

The lysostaphin used contained only about 30% free N-terminal threonine. Conditions of Gaertner & Offord were used for oxidation of the N-terminal threonine. In more detail, a 50 molar excess of methionine (17.5  $\mu$ l from a 1M stock in water) was added to 1 ml of a 10 mg/ml solution of lysostaphin. Sodium bicarbonate (1M) was added to adjust the pH to 8.3. Oxidation was commenced  
15 by the addition of sodium periodate (7  $\mu$ l from a 0.5 M stock in water). The reaction mixture was kept in the dark at room temperature for 10 minutes, at which time 7.1 mg Bis(amino-oxy)tetraethylene glycol (obtained from Solulink™) prepared as a 50 mg/ml solution in DMSO was added. After 1 hour in the dark,  
20 the solution was dialyzed against saline in the dark at room temperature. The product is termed lysostaphin AO. The lysostaphin concentration was determined at OD 280 using 0.49 mg/ml/Absorbance unit.

An aliquot was tested with TNBS at pH 5. It has previously been found that amino-oxy but not amines reacted with TNBS under these conditions. The assay  
25 was performed as follows: 50  $\mu$ l of lysostaphin or lysostaphin AO was added to 440  $\mu$ l of 0.1 M NaAc, pH 5 and then 10  $\mu$ l of 10 mg/ml TNBS in water added. 5  $\mu$ l of 1 mM Amino-oxy acetic acid was used as a standard in the above solution.



Samples were read at 500 nm after a 6 hour incubation in the dark. The sample solution was orange, indicating presence of amino-oxy groups. Using the standard, it was estimated about 30% of lysostaphin was derivatized with the AO group. This lysostaphin AO was then reacted with excess oxidized T2000 dextran in the dark at room temperature to allow conjugation via oxime formation. The reaction was assayed by SEC HPLC to determine the shift of mass from low molecular weight (unconjugated protein) to high molecular weight (lysostaphin-dextran conjugate). A Phenomenex Biosep SEC2000 (300x4.6) equilibrated with PBS and run at 0.5 ml/min with monitoring at 280 nm was used for SEC HPLC

With reference to Figure 6, the upper chromatogram is the reaction mixture at about 1 minute, the middle chromatogram is after an overnight reaction and the lower figure is the lysostaphin AO alone. Note the shift to high molecular weight material after the reaction was allowed to proceed overnight. This figure suggests that the AO group on the lysostaphin linked to the high molecular weight, oxidized dextran. About 27% was coupled, based on the percentage of the area of the high molecular weight peak. This is in the expected percentage since only about a third of the lysostaphin contained a free threonine and was derivatized with AO, as indicated by TNBS assay.

#### **Example 19: Preparation of DT(ox)-AO-Pn14 Conjugate**

This example illustrates the preparation of the DT(ox)-AO-Pn14 conjugate, and it also demonstrates how reagents can be prepared in as "single pot" reactions (which may simplify preparation).

##### I. Mercaptoglycerol-Diphtheria toxoid

0.5 ml diphtheria toxoid (~13 mg/ml) was combined with 100  $\mu$ l 1 M HEPES, pH 8 and 10  $\mu$ l 0.1 M NHS bromoacetate in NMP. It was incubated in the dark for about 30 minutes, and then 10  $\mu$ l of 12.3  $\mu$ l mercaptoglycerol was added.

Following an overnight reaction, the solution was desalted with an Amicon Ultra 4 (30kDa cutoff) to a final volume of about 400  $\mu$ l.

Next, 50  $\mu$ l of 1 M sodium acetate at pH 5 was added, followed by 9  $\mu$ l of 0.5 M sodium periodate. Oxidation was allowed to proceed for 10 minutes in the dark at room temperature. The reaction was then quenched by the addition of 50% glycerol. The low molecular weight components were removed on the same Amicon Ultra 4 device and diafiltered into saline. The final volume was about 200  $\mu$ l.

The above protocol eliminated one of the desalting steps by adding excess mercaptoglycerol to the solution containing bromoacetylated-DT and bromoacetate.

## II. Conjugation

1 ml of amino-oxy Pn14 (~9 mg/ml) was added to the oxidized DT and 100  $\mu$ l of 1 M sodium acetate at pH 5 added. The reaction was allowed to proceed overnight at room temperature in the dark, and then fractionated on an S400HR column (1x60 cm, equilibrated with saline).

The high molecular weight fraction was pooled. Protein was estimated using 1 mg/ml = 1 OD and the Pn14 concentration determined using the resorcinol assay. The fraction was found to contain about 1.3 mg DT/mg Pn14.

### Example 20: Preparation of a gp350(ox)-AO-S-Pn14 conjugate.

gp350 is a glycoprotein from Epstein Barr virus that binds to human complement receptor. It was produced recombinantly in yeast cells by Dr. Goutam Sen (Uniformed Services University of the Health Sciences, Bethesda, MD) and purified by hydrophobic interaction chromatography.

The pH of 0.5 ml of gp350 at 8 mg/ml in PBS was reduced by the addition of 50  $\mu$ l 1 M sodium acetate, pH 4.7, and 11  $\mu$ l of 0.5 M sodium periodate (in water) was added. After an 8 minute incubation in the dark, on ice, the reaction